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Short communication

The sensitivity of *Plasmodium* protein synthesis to prokaryotic ribosomal inhibitors

Ariati S. Budimulja[a], Syafruddin[a], Pramuan Tapchaisri[b], Prapon Wilairat[c], Sangkot Marzuki[a,*]

[a]*Eijkman Institute for Molecular Biology, Jl. Diponegoro 69, Jakarta 10430, Indonesia*
[b]*Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand*
[c]*Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand*

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The prokaryotic protein synthesis inhibitors tetracycline and doxycycline are used in the treatment of malaria, as a way to overcome the problem of drug resistance to more conventional antimalarial drugs. It has been suggested that the antimalarial activity is mediated through the inhibition of the protein synthetic machinery of the mitochondria [1], as suggested by its sensitivity in a wide range of eukaryotic organisms to inhibitors of the 70S prokaryotic ribosomes; these inhibitors do not normally inhibit the main protein synthesizing machinery in the cytosol [2,3].

Several recent findings, however, have prompted us to examine the sensitivity of the *Plasmodium* protein synthesis to these inhibitors and to question the mechanism of action of the prokaryotic protein synthesis inhibitors as antimalarial drugs. Most notable is the observation that the malaria parasite has a 35 kb circular extrachromosomal DNA of plastid origin [4] which has led to interesting questions with regard to the *Plasmodium* place in the evolutionary tree, in particular in view of an earlier suggestion that the ribosomes of protozoa might not in all cases fit into the dichotomous prokaryote-eukaryote (70S vs. 80S) classification [5]. The appropriateness of the dichotomous classification in general has been questioned [6,7], and the ribosomes of protozoa suggested to occupy an intermediate position.

Differing from previous studies [8–12], we have measured carefully the initial rate of the protein synthetic activity in the presence of a wide range of antibiotic concentrations, allowing the construction of proper dose-dependent inhibition

* Corresponding author. Tel.: + 62 21 3148694; fax: + 62 21 3147982; e-mail: smarzuki@eijkman.go.id

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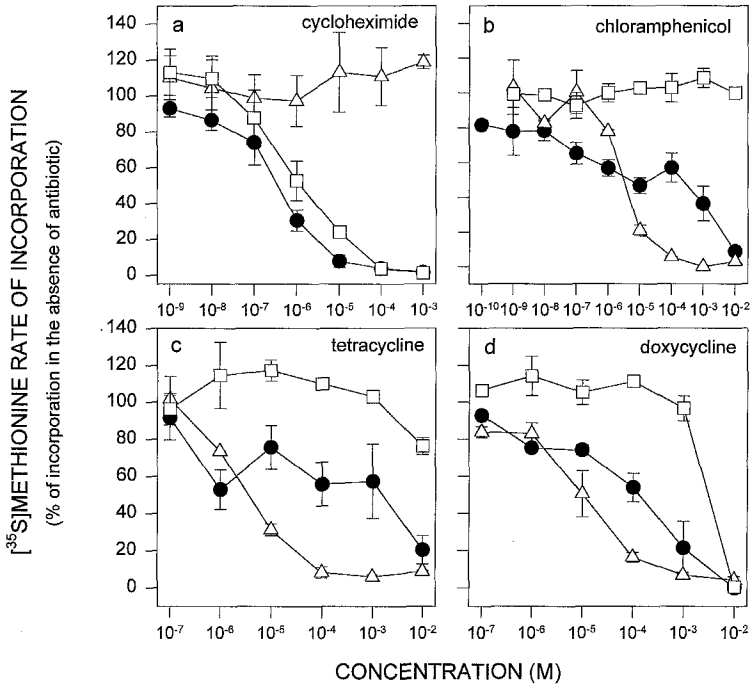

Fig. 1. Sensitivity of *P. falciparum* (●), *S. cerevisiae* (□) and *E. coli* (△) protein synthesis to inhibitors of the 70S and 80S ribosomes. *P. falciparum* (FCR-3 strain), cultured in vitro [19], was harvested at the parasitemia level of ≈ 10%, predominantly of early and late trophozoite stage. Infected red blood cells were resuspended in methionine-free RPMI-1640 medium (Gibco BRL, Selectamine Kit) supplemented with 25 mM HEPES buffer and 2 g/L $NaHCO_3$ (final volume 1 ml) to a final concentration of 1×10^7 parasites/ml, containing cycloheximide, emetine, chloramphenicol, tetracycline or doxycycline at the indicated concentrations. After preincubation for 5 min at 37°C in an atmosphere of 5% CO_2 and 20% O_2, with gentle shaking in a gyratory shaker, 5 μl of the Expre^{35}S^{35}S protein labeling mix (Du Pont; 50 mCi/tube, specific activity 1175 Ci/mmol) was added. At intervals (0, 10, 20, 30, 40, 50 and 60 min), 100 μl aliquots were taken and immediately added into microfuge tubes containing ice cold RPMI-1640 medium with excess methionine (1 mg/ml). Parasites were released using 0.2% (w/v) saponin [20], collected by centrifugation at 1150 × *g* at room temperature and resuspended in 1 ml of 7% trichloroacetic acid containing 1 mg/ml methionine. Protein precipitate was transferred onto nitrocellulose membranes (Bio-Dot Microfiltration Apparatus, Bio-Rad), and washed three times with 7% trichloroacetic acid. The nitrocellulose membranes were air-dried and impregnated with solid scintillator (Microbeta™ 1450-441 MeltiLex, Wallac, Finland), for the determination of the radioactivity (1450 Microbeta™ PLUS, Wallac, Finland). The initial rates of protein synthesis were determined from the linear part of the time course. Results are expressed as the percent of the rate of incorporation in the absence of inhibitor (ranging from 1300 to 2000 dpm/min per 10^{-7} cells), and are the mean ± S.D. of 3–6 independent experiments. The inhibition curves of *S. cerevisiae* (BSC 483/1a strain) and *E. coli* (JM 101) were examined essentially as for *P. falciparum* except that a yeast nitrogen base (0.67%) medium (DIFCO Laboratories) supplemented with 5 g/L D-glucose and 5 mg/L of all amino acids besides methionine, was used for the protein synthesis of *S. cerevisiae* (1 mg cell dry weight/ml), and methionine assay medium (DIFCO Laboratories) for *E. coli* (2×10^7 cells/ml).

curves. Intraerythrocytic parasites incorporated [^{35}S]methionine into trichloroacetic acid-insoluble material linearly for at least 60 min (data not shown). Its sensitivity to the 80S ribosomes inhibitor, cycloheximide, could be demonstrated clearly, and a typical drug inhibition curve was observed (Fig. 1a) with an IC_{50} of around 5×10^{-7} M. An almost similar inhibition curve was observed when the protein synthetic activity of the yeast *S. cerevisiae* was examined. In contrast, cycloheximide did not inhibit the incorporation of [^{35}S]methionine by the prokaryotic *Escherichia coli* cells, even at very high concentrations (up to 10^{-3} M). A similar inhibitory effect was also obtained for emetine (IC_{50} 10^{-7} M), another inhibitor of the 80S ribosomes (data not shown).

When the protein synthesis activity of the *Plasmodium* parasite was similarly tested against the 70S ribosomal inhibitors (chloramphenicol, tetracycline and doxycycline), the activity was found to be inhibited also in a dose dependent manner (Fig. 1b–d). However, in contrast to that observed for *E. coli* (Fig. 1b–d) the inhibition curves did not follow a typical sigmoidal drug inhibition pattern and thus the IC_{50} could not be determined precisely (between 7×10^{-6} and 2×10^{-5} M for chloramphenicol, 10^{-6}–10^{-4} M for tetracycline and around 10^{-4} M for doxycycline). Rather, the curves suggest the possibility of biphasic inhibition. This inhibition is not an artefact, as the protein synthetic activity of a typical eukaryote, the yeast *S. cerevisiae*, is not affected at much higher concentrations of the inhibitors.

Our results are consistent with the suggestion that the *Plasmodium* ribosomes do not fit into the prokaryote-eukaryote (70S vs. 80S) classification. It has been suggested also that the ribosomes of *Eimeria tenella*, a protozoan parasite of domestic animals, have some prokaryotic characteristics [13] and its protein synthetic activity in vitro is sensitive to inhibition by tetracycline and chloramphenicol. Results of several previous drug inhibition studies are indeed in agreement with our suggestion. Sherman et al. [8] reported that chloramphenicol at 4×10^{-3} M inhibited [^{14}C]isoleucine incorporation by *P. lophurae* (cycloheximide gave 90% inhibition at 2×10^{-5} M). Poly(U)-directed synthesis of polyphenylalanine by isolated ribosomes of *P. knowlesi* was inhibited ($>95\%$) by 10^{-4} M chlortetracycline [10]. Further support came from the analysis of the sequence of the *Plasmodium* rRNAs for potential sites of interaction with antibiotics [14], which predict on the basis of equivalent nucleotides observed in sensitive or resistant strains of *E. coli*, that *Plasmodium falciparum* ribosomes should be sensitive to chloramphenicol.

Some previous studies arrived at conclusions in contrast to our results [8,9]. However, these studies were carried out with prolonged incubation periods (from 7 to 27 h, with up to 3 h preincubation prior to the addition of the radioactively labelled amino acid tracer). Such an experimental set up does not allow the proper measurement of the rate of protein synthesis, as the rate of the amino acid incorporation is the manifestation of the balance between protein synthesis and degradation; the latter presumably stimulated by the prolonged incubation in the presence of inhibitors.

What is the explanation for the untypical inhibition curves of the *P. falciparum* protein synthesis by the prokaryotic ribosomal inhibitors? One possibility is that the curves might in fact be the manifestation of mixed populations of ribosomes which have dissimilar sensitivity to the 70S ribosomal inhibitors. There are indeed reports in the literature that support this possibility. Each haploid genome of *P. falciparum* contains six copies of rRNA gene sets [15], expressed in a stage-specific manner [16] during the parasite development in the mosquito vector and vertebrate host. The switching of ribosome types occurs during the development process, between the S (sporozoite; sexual), A (asexual) and O types of ribosomes. In the erythrocytic stage most of the ribosomes are of the A type. However, three copies of type A rRNA genes (and two copies of type S) have been found in *P. falciparum* [15]; uniquely, these rRNA genes are dispersed in as many chromosomes and evolved independently. An analysis of the structure of the various large subunit rRNAs comprising the type A ribosomes might give indications of differences in drug sensitivity.

Since the inhibition curves of the 70S ribosomal inhibitors suggested the possibility of a biphasic inhibition (for chloramphenicol the IC_{50} are 10^{-7} and 10^{-3} M for the first and second phase of inhibition respectively), we have also examined whether the spectrum of protein products inhibited in the initial phase of inhibition is similar to that in the later phase. No qualitative differences could be observed in the pattern of protein synthesis products in the presence of various concentrations of chloramphenicol when the [^{35}S]labelled products were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (data not shown; similar results were obtained also with tetracycline and doxycycline). However, more subtle differences in the protein synthesis products would be undetectable by SDS-polyacrylamide gel electrophoresis (SDS-

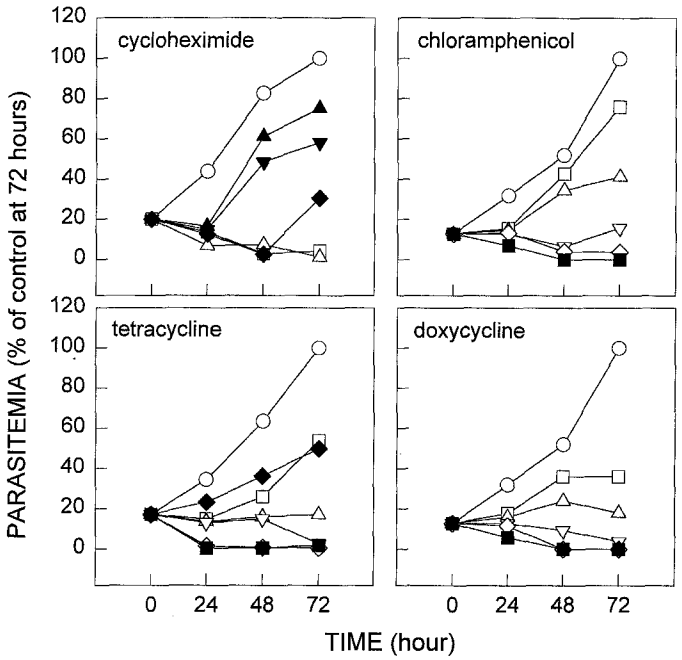

Fig. 2. Effect of 80S and 70S ribosomal inhibitors on the in vitro growth of *P. falciparum*. The sensitivity of the parasite to cycloheximide, chloramphenicol, tetracycline and doxycycline was examined in vitro in 24 well plates. Each well contained (in 1 ml) the growth medium (RPMI supplemented with 5% human serum of the AB type, 25 mM HEPES and 2 g/L $NaHCO_3$), RBC (10% haematocrit) and different concentrations of inhibitors as specified. The initial parasitemias were 0.3–1% of young trophozoites. Cultures were incubated at 37°C in an atmosphere of 5% CO_2. The growth medium was changed every day and the degree of parasitemia was determined at the time intervals indicated in the absence (○) or the presence of an inhibitor at the concentrations of 10^{-9} M (▲), 10^{-8} M (▼), 10^{-7} M (◆), 10^{-6} M (□), 10^{-5} M (△), 10^{-4} M (▽), 10^{-3} M (◇), 10^{-2} M (■).

PAGE). A more definitive study on the functional significance of the above observation would require the analysis of the products of protein synthesis by two dimensional gel electrophoresis.

We could not obtain evidence on the sensitivity of the *Plasmodium* mitochondrial protein synthesis to the prokaryotic protein synthesis inhibitors. First, cycloheximide and emetine, which in other organisms do not affect mitochondrial protein synthesis, were found to inhibit the total *Plasmodium* protein synthesis almost completely (Fig. 1a); there was only trace levels of cycloheximide- or emetine-insensitive [^{35}S]methionine incorporation detectable, indicating the low activity of mitochondrial protein synthesis (assuming that the *Plasmodium* mitochondrial protein synthesis is insensitive to inhibition by cycloheximide). Kiatfuengfoo et al. [12] reported the continuing synthesis of two proteins (95 and 85 kDa) by *P. falciparum* in the presence of cycloheximide (10^{-4} M) and suggested that these proteins are products of mitochondrial protein synthesis. While this could be the case, it should be noted that prolonged inhibition of the cytoplasmic protein synthesis would eventually affect mitochondrial protein synthesis as well; all mitochondrial ribosomal proteins and other factors of the protein synthetic machinery are synthesized in the cytoplasm. The inhibition time in the above study [12] was 7 h, while in many organisms such as the yeast *S. cerevisiae* and human, mitochondrial protein synthesis ceases within 1—2 h following the inhibition of cytoplasmic protein synthesis [3].

Results of our in vitro growth inhibition experiment (Fig. 2), which are in agreement with those of previous studies [1,17,18], are consistent with a direct effect of the prokaryotic protein synthesis inhibitors on the cytoplasmic ribosomes, rather

than an effect through the inhibition of mitochondrial protein synthesis. Thus, chloramphenicol, tetracycline and doxycycline were found to inhibit the in vitro growth of *P. falciparum* within 24–72 h of exposure, similar to the pattern of inhibition by the 80S ribosomal inhibitor, cycloheximide. Growth inhibition mediated by an effect on the mitochondrial protein synthesis is characterized by a delayed inhibition pattern; the cellular turnover of products of mitochondrial protein synthesis is relatively low and thus these products which are essential for oxidative energy metabolism, have to be diluted first to a critical level by cell divisions before any effect on growth could be observed [3].

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